THE EMBO JOURNAL

Manuscript EMBO-2011-78853

Myeloid Translocation Gene 16 is required for maintenance of hematopoietic stem cell quiescence

Melissa A Fischer, Isabel Moreno-Miralles, Aubrey Hunt, Brenda J Chyla and Scott W Hiebert

Corresponding author: Scott W. Hiebert, Vanderbilt University

Review timeline:

Submission date:	18 July 2011
Pre-Decision Letter:	26 August 2011
Authors' Response:	30 August 2011
Editorial Decision:	01 September 2011
Revision received:	02 November 2011
Accepted:	16 December 2011

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Pre-Decision Letter 26 August 2011

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below.

You will see that while the referees find your study of potential interest they do raise significant concerns. As their reports are explicit, I would like to highlight here the most critical issues:

1- The niche contribution of the observed phenotype should be ruled out (ref1 point1)

2- A better-defined population of LT-HSC should be used (ref1 point3, ref2 point3, ref3 point5) to ascertain the quiescence is really conferred by LT-HSC, without any participation of short term-HSCs

3- Analysis of progenitors in myeloid and lymphoid lineages should be done (ref1 point5 and ref2 point4) to ascertain that the lethality observed comes from SC failure

While we recognize that addressing points 2 and 3 should be feasible, we are more concerned as to whether and how you may be able to respond to point1, particularly since it is clear that the experiments suggested would go beyond the scope of a normal major revision.

Before deciding how to proceed, it would be useful to have your response to all concerns raised by the referees in general and to the abovementioned point 1, in particular. If you could reply to the

referees' concerns point-by-point with your comments, in terms of whether you can address the issues and a rough time line, this would help us reaching a fair and timely decision.

Thank you for the opportunity to consider your work for publication. I look forward to your reply.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

The authors here use an Mtg16 knockout mouse strain to address the role of Mtg16 in hematopoietic stem cell function. It is observed that HSCs, and in particular immature HSCs (LSKCD150+CD48-), are under-represented by phenotype in Mtg16 null bone marrow. These cells are almost entirely lost during successive competitive transplantations. This is attributed to increased cycling of HSCs, as measured by BrdU incorporation and Hoechst/Pyronin Y staining of LSK cells. Gene expression profiling of wild type and mutant LSK populations identified up-regulation of several positive regulators of cell cycle progression (Mycn, Ccnd1, E2f2) of which E2f2 was found to bind Mtg16 by ChIP analysis.

While the results presented clearly identify a defect in repopulation of HSCs derived from Mtg16 null mice, it is not clear that the conclusion that this is a defect intrinsic to the HSC compartment is fully supported by the data, not is it clear that the cellular defect identified, namely increased cycling, can be attributed to repopulating HSCs. Finally, the putative role of deregulation of cell cycle genes in the observed phenotype is not substantiated by any experimental data.

Major points:

1. A general issue with assessing the role of intrinsic regulators of adult definitive HSC function using a general (as opposed to conditional) knockout is that the effect on the HSCs may be indirect (e.g. through a role for the gene, here Mtg16, in the developmental or adult HSC niche), or developmental (e.g. due to an intrinsic defect in formation of fetal liver HSCs, leading to an exhaustion phenotype in the adult HSCs). Some, but not all, of these concerns could be addressed by the reconstitution of Mtg16 null recipients with wild type bone marrow, and assessing function of HSCs some months post-transplant through re-transplantation into wild-type recipients, which would provide information about the state of the HSC niche in adult Mtg16 null mice. However, the state of the art for this type of experiment is the inactivation of a conditional allele in transplanted bone marrow, whereby developmental and niche phenotypes can be circumvented.

These considerations are not entirely theoretical: while the data are not fully comparable, Mtg16-deficient HSCs do not decrease as a percentage of the total Mtg16 BM population relative to wild type HSCs when transplanted into a wild-type host (Figure 2D), in seeming contrast to what is observed in an Mtg16 null host (Figure 1), indicative of an environmental contribution to the phenotype.

2. A distinction needs to be made between abundance and function of repopulating stem cells. Given the finding that primitive HSCs are reduced >75% in the Mtg16 null bone marrow, a more precise assessment of the function of these HSCs would be obtained through transplantation of equal numbers of sorted wild type and mutant CD45.2 LT-HSCs along with CD45.1 competitor bone marrow. Also, the part of Figure 2B where reconstitution at the zero time point is shown seems unlikely to be an actual experimental measurement, and, if it is not, should be removed from the graph.

3. To identify a defect in maintenance of HSC quiescence it is necessary to study the quiescent fraction of the HSC compartment, the LT-HSCs. For this purpose, the LSK or LSKFlt3- phenotypes are not sufficient. An acceptable phenotype for this purpose would be LSKCD150+CD48- (or even

better: LSKCD150+CD48-CD34-). The current data analyze cell populations, which are overwhelmingly composed of short term HSCs with cycling properties completely different from repopulating HSCs. Given that perhaps only 2-3% of LSK cells are true LT-HSCs, a decrease in LSK quiescence from 30 to 25% does not allow any conclusions to be reached regarding the LT-HSC subset.

4. The experiment showing normal homing of Mtg16 null bone marrow cells does not address whether the cells found in the bone have a stem cell phenotype. It should be possible to determine at least the number of LSK cells by flow cytometry. For the colony assays on homed cells, one would of course expect the un-injected background to be zero colonies, but it would probably be appropriate to verify this experimentally.

5. The failure of Mtg16 null bone marrow to rescue lethally irradiated recipients need not necessarily be due to stem cell failure. The authors have themselves previously shown that Mtg16 null bone marrow shows a myeloid-biased lineage allocation. It is therefore entirely possible that lethality is, at least to some extent, due to failure to adequately reconstitute erythrocytes and platelets, but not a general hematopoietic failure. Analysis of the peripheral blood (erythrocyte parameters, platelet numbers and differential counts would suffice) during the recovery phase would allow the relative kinetics of myeloid and platelet/erythrocyte reconstitution to be assessed.

6. The analysis of the gene expression data needs to be more systematic and unbiased. For example, is the enrichment of S-phase genes systematic and significant? Gene Ontology or Gene Set Enrichment Analysis-based interpretations would be appropriate. The identification of E2f2 as a putative Mtg16 target in interesting, but no functional data is provided to assess its relevance (shRNA-based lentiviral knockdown in Mtg16 null HSCs, for example, would be fairly straightforward, and should even benefit from a selective advantage of the knockdown cells, if the authors' hypothesis is correct).

Minor points:

1. Gene nomenclature is highly inaccurate in many places; it is not generally possible to obtain a correct gene name simply by italicizing the protein name (Cyclin D1 = Ccnd1, PU.1 = Sfpi1, Neurofibromatosis-1 = Nf1, C/EBPa = Cebpa, EpoR = Epor, N-Myc = Mycn etc).

2. For competitively repopulation assays it would be useful if the lineage allocation (B, T, myeloid) of the CD45.1 and CD45.2 fractions could be provided. Since lymphoid and myeloid cells repopulate and decay with very different kinetics differences in lineage allocation may affect the interpretation of the results.

3. For the microarray analysis the methodology used for quality control and data processing should be explained, and an accession number for the primary data provided.

4. The gating for lack of Flt3 expression seems to include a very small number of cells, compared to what is normally considered Flt3- in the LSK compartment (35-40% of LSK cells).

5. Indicating the size of gated populations on the flow cytometry plots would be helpful.

Referee #2

In this manuscript, the authors report an essential function for the transcriptional repressor Myeloid Translocation Gene 16 (MTG16) in the maintenance of hematopoietic stem cells (HSCs). A previous paper from this group had described the generation of MTG16-deficient mice (Chyla et al., MCB 2008), as well as specific hematopoietic defects in these mice (erythroid differentiation, bone marrow B lineage cells, CFU-S8, CFU-S12). The current manuscript provides a much more detailed description of the HSC compartment in these mice. They show that Mtg16 loss results in decreased numbers of phenotypically defined HSCs in the bone marrow, markedly decreased radioprotection capacity and decreased long-term reconstitution potential in competitive repopulation assays. Homing to the bone marrow appeared to be preserved, at least when crudely assessed 16 hours after transplantation. In vitro assays showed loss of replating potential and decreased colony formation in

LTC-IC assays, suggesting decreased self-renewal. Cell cycle and BrdU incorporation analysis revealed an increased proportion of MTG16-deficient progenitors in S phase and a decrease proportion in G0, suggesting decreased quiescence. Finally, gene expression analysis revealed several upregulated genes that could contribute to the hematopoietic phenotype after loss of MTG16-mediated repression (including N-myc, Cyclin D1, E2F2 and Id family members). In the case of E2F2, this was correlated with ChIP data showing recruitment of both E47 and MTG16 to the E2F2 locus (suggesting that MTG16 could function as a repressor by associating with E proteins at this locus).

Altogether, this is an interesting paper that reports new information about MTG16. In particular, the characterization of HSC defects in MTG16-deficient mice is done very carefully. The authors provide several suggestions regarding the mechanistic effects of MTG16 loss, largely based on gene expression arrays, with more detailed investigation by ChIP of a small subset of candidate targets. Given the possible interaction of MTG16 with multiple transcriptional partners, definitive mechanistic analysis will likely involve a large amount of work.

Specific comments:

1) Figure 1: it would be very useful for the reader if percentages of events were indicated for the gating boxes in the dot plots (even if absolute cell numbers are represented by bar graphs). This applies to other dot plots across the manuscript.

2) Figure 6C: ChIP analysis is performed in a cell line rather than in primary bone marrow cells. Although there are obviously technical arguments to support this approach, it would be interesting to know if recruitment of Mtg16 and E47 to the E2F2 locus could be detected in primary cells. Also, can the authors evaluate if Id proteins were also recruited to this locus? This would also have the potential to repress the effects of E proteins, especially given elevated Id1/2 expression in MTG16-deficient cells.

3) Figure 7, S5: a central claim in the manuscript is the decreased quiescence of MTG16-deficient long-term HSCs. Thus, it would be very useful if analysis of quiescence and cell cycle activity were performed on as strictly defined populations of HSCs as possible (rather than on heterogenous LSK cells). Fig. S5C is helpful by gating on Flt3- LSK cells and could be shown as a main figure. Even better would be to analyze HSCs as defined in figure 1 using LSK staining and SLAM markers.

4) Figure S1: although described as showing enhanced myeloid production, the findings could equally be explained by reduced representation of B lineage cells in the MTG16-deficient bone marrow. In other words the increase in myeloid cells could only be relative. The authors need to reword their description. Analysis of lymphoid and myeloid progenitors would also be helpful to further document this phenomenon.

5) Table S1: upregulated genes include several genes that have been described as Notch target genes (Hes1, Nrarp, Id1, among others). The authors have previously published work showing an interaction between CSL and MTG16. Can the authors elaborate on that? Can some of the gene expression changes observed in MTG16-deficient progenitors be related to loss of CSL/MTG16-mediated repression of Notch target genes?

Referee #3

The manuscript by Fisher et al reports the finding that Mtg16 is required to maintain hematopoietic stem cells in quiescence and to control their long term self-renewal capacity. The authors show that Mtg16^{-/-} HSCs fail to reconstitute hematopoiesis both in serial and in competitive transplantation assays whereas their marrow homing capacities remain unaltered, consistent with a cell-intrinsic defect. By BrdU labelling and Hoechst staining, the authors identify a defect in the quiescence state of the LSK population which is enriched in HSC, associated with increased E2f2 expression.
Comments:
In adult mice, hematopoietic stem cells reside in a quiescence state that preserves their long term activity. A number of genes have been shown to control this quiescence state and the authors

provide additional evidence for a role of the Mtg16 co-repressor in this process. The work is carefully conducted. Although primary transplantation assays show that Mtg16^{-/-} cells are less competitive and are prematurely exhausted, secondary transplantation is more revealing of long term stem cell activity and the most convincing data come from the assessment of the population of LSK/Flt3- which is severely decreased in secondary transplantation (Figure 3B). Nonetheless, a number of issues compromise the manuscript in its current form and should be addressed.

1) Given that the read out in transplantation assays is the percentage of donor-derived mature cells and given that the authors have previously shown an important role for Mtg16 in short term stem cells and multipotent progenitors, the question arises whether the decreased competition that the authors report in the present study might be due to decreased short term stem cell/multipotent progenitors or decreased long term stem cells. Indeed, the early lethality of mice transplanted with Mtg16_{-/-} cells at 30-40 days is a clear indication of a failure of short term reconstitution (Figure 2A).

2) The authors have circumvented this early lethality problem by transplanting a limiting dose of normal bone marrow cells at a ratio of 1:10. This approach clearly solved the lethality problem but does not directly address the question of long term stem cell self-renewal even if normal cells outcompete Mtg16_{-/-} cells, because of the above mentioned possibility that the read out depends not only on long term stem cells but also on progenitors that are continuously generated from these stem cells. Unless the authors can find conditions in which short term stem cells and progenitors are not compromised while long term stem cells are significantly affected, then the conclusions of the study need to take into consideration the confounding issues of short term versus long term stem cell activity.

3) Along the same line, in vitro serial replating and long term culture with exhaustion at 3 weeks illustrated in Figure 5 are also compatible with more short term stem cell activity.

4) The authors show by various approaches that E2f2 is a target of regulation by Mtg16. However, the functional significance of E2f2 upregulation in this study is unclear. Is the phenotype reported here for Mtg16 deficiency rescued by knocking down E2f2?

5) Finally, quiescence monitored in the LSK population shows rather modest variations between Mtg16<sup>+/+</sub> and Mtg16-/- cells. Have the authors monitored cell cycle in subpopulations that are more enriched in long term stem cells, i.e. SLAM/LSK or LSK/Flt3^{neg}?

Authors' Response 30 August 2011

Thanks again for allowing us to respond to the reviewer's comments. After careful consideration, we believe that by adding only a couple of pieces of data that we have in hand, doing some short term studies, and revising the text to clarify some issues for the reviewers, we will be able to respond to each and every concern raised by the reviewers. In fact, for most of Reviewer #1's comments the data were in the paper, but we probably did a poor job of describing it. I appreciate the difficulty of assessing such a paper and recognize that I might have made similar requests before going through the pitfalls of this stem cell analysis over the past 4 years. For example, the reviewer asks us for a FACS experiment to assess homing in point #4 stating that "It should be possible to determine at least the number of LSK cells by flow cytometry". I had the same idea and we naively tried this experiment as our first assessment of homing years ago and quickly realized that it is impossible because 30,000,000 cells are in the process of dying due to the radiation and you are trying to identify 0.01% of the live cells. FACS just doesn't work in this background and one has to assess homing using methylcellulose colony formation assays (as we did in Fig. 4). Thus, while most of the 1st reviewer's comments are just a bit naive, we value these comments as they point out areas that we need to clarify the text to make it more understandable. Attached is a full response to the reviewers laying out some new data along with proposed new experiments. We hope that you will agree that the new data included (Fig. 5C), better explanations, and the proposed experiments will address these concerns.

1- The niche contribution of the observed phenotype should be ruled out (ref1 point1)

We too were concerned about niche contributions and we did several experiments to address this issue. First, *Cdc42*$^{-/-}$ mice are perhaps the most famous case of a niche defect and these mice lose cell adhesion, allowing stem cells to escape the marrow. Therefore, we used both FACS and methylcellulose colony forming assays to determine if more stem cells were mobilized to the peripheral blood in our mice. Neither assay showed any change. We will modify the text to make this point. Second, we have transplanted wild type marrow into null mice. Because there were no obvious changes in hematopoiesis at 12 weeks post transplant (as compared to the changes we observed with null marrow into wild type recipient mice with wild type niche), we terminated the experiment. Third, a possible niche contribution was one of the reasons for performing the in vitro analysis (LTC-IC and methylcellulose replating assays) that take null cells out of the niche and assess the action of the stem cells outside the animal (for LTC-IC on wild type stroma). *Cdc42*$^{-/-}$ mice show no defects in these assays, while Mtg16-null stem cells do.

We also propose to add data showing that we can re-express Mtg16 in null bone marrow cells and complement the defects in LTC-IC assays. If the defects were due to Niche defects, this complementation would not work. Finally, we have yet to find a phenotype in Mtg16$^{-/-}$ murine embryonic fibroblasts. In fact, Mtg16 is expressed at much higher levels in LSK and B cells than any other hematopoietic cells or fibroblasts (Hunt et al., 2011, Supplemental figure 3 and data not shown), whereas Mtg8 is the key MTG family member in fibroblasts. We are currently testing the levels of expression of Mtg16 in bone marrow stromal cells and expect to see little Mtg16 expressed. This assay should not take more than a week or so to complete and could be added to the data in this manuscript.

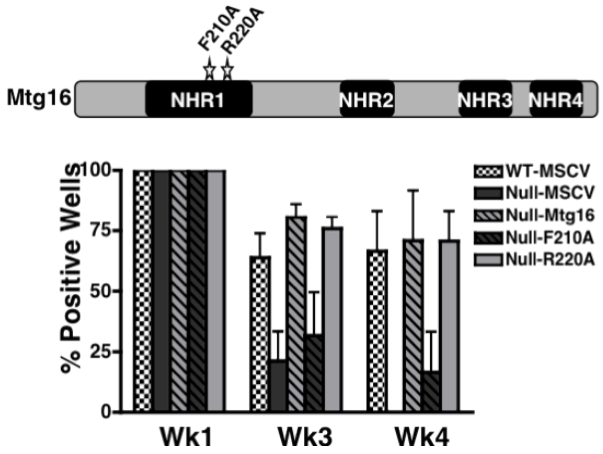

Fig. 5C. Mtg16 re-expression, but not Mtg16(F210A), complements the LTC-IC defect in Mtg16-null bone marrow cells. Schematic diagram shows the positions of the F210A (blocks Mtg8 binding to HEB and Mtg16 suppression of E protein-dependent transcription) and R220A (control mutation that does not affect E protein binding in the crystal structure of Mtg8).

2- A better-defined population of LT-HSC should be used (ref1 point3, ref2 point3, ref3 point5) to ascertain the quiescence is really conferred by LT-HSC, without any participation of short term-HSCs

Though it does get more technically difficult to look at these small populations (due to the addition of so many fluorochromes that bleed into the adjacent channels and such small numbers of cells), we can certainly look at the proliferation status in the LSK/SLAM population (since we did already examine the LSK/Flt3 population). This assay could be completed in a week.

3- Analysis of progenitors in myeloid and lymphoid lineages should be done (ref1 point5 and ref2 point4) to ascertain that the lethality observed comes from SC failure

We have these data in hand, but the point of the non-competitive bone marrow transplant was simply to introduce the need for competitive transplants to assess the function of the LT-HSC. The competitive transplant is the gold standard as it measures the function of the LT-HSC in the context of wild type progenitor and mature cells. We took this analysis out to one year, which is 40 weeks longer than most investigators have done (most experiments are terminated at 12 weeks).

Referees'comments:

Referee #1

(Remarks to the Author)

The authors here use an Mtg16 knockout mouse strain to address the role of Mtg16 in hematopoietic stem cell function. It is observed that HSCs, and in particular immature HSCs (LSKCD150+CD48-), are under-represented by phenotype in Mtg16 null bone marrow. These cells are almost entirely lost during successive competitive transplantations. This is attributed to increased cycling of HSCs, as measured by BrdU incorporation and Hoechst/Pyronin Y staining of LSK cells. Gene expression profiling of wild type and mutant LSK populations identified up-regulation of several positive regulators of cell cycle progression (Mycn, Ccnd1, E2f2) of which E2f2 was found to bind Mtg16 by ChIP analysis.

While the results presented clearly identify a defect in repopulation of HSCs derived from Mtg16 null mice, it is not clear that the conclusion that this is a defect intrinsic to the HSC compartment is fully supported by the data, not is it clear that the cellular defect identified, namely increased cycling, can be attributed to repopulating HSCs. Finally, the putative role of deregulation of cell cycle genes in the observed phenotype is not substantiated by any experimental data.

Major points:

1. A general issue with assessing the role of intrinsic regulators of adult definitive HSC function using a general (as opposed to conditional) knockout is that the effect on the HSCs may be indirect (e.g. through a role for the gene, here Mtg16, in the developmental or adult HSC niche), or developmental (e.g. due to an intrinsic defect in formation of fetal liver HSCs, leading to an exhaustion phenotype in the adult HSCs). Some, but not all, of these concerns could be addressed by the reconstitution of Mtg16 null recipients with wild type bone marrow, and assessing function of HSCs some months post-transplant through re-transplantation into wild-type recipients, which would provide information about the state of the HSC niche in adult Mtg16 null mice. However, the state of the art for this type of experiment is the inactivation of a conditional allele in transplanted bone marrow, whereby developmental and niche phenotypes can be circumvented.

These considerations are not entirely theoretical: while the data are not fully comparable, Mtg16-deficient HSCs do not decrease as a percentage of the total Mtg16 BM population relative to wild type HSCs when transplanted into a wild-type host (Figure 2D), in seeming contrast to what is observed in an Mtg16 null host (Figure 1), indicative of an environmental contribution to the phenotype.

We too were concerned about niche contributions and we did several experiments to address this issue. First, *Cdc42*$^{-/-}$ mice are perhaps the most famous case of a niche defect and these mice lose cell adhesion, allowing stem cells to escape the marrow. Therefore, we used both FACS and methylcellulose colony forming assays to determine if more stem cells were mobilized to the peripheral blood in our mice. Neither assay showed any change. We will modify the text to make this point. Second, we have transplanted wild type marrow into null mice. Because there were no obvious changes in hematopoiesis at 12 weeks post transplant (as compared to the

changes we observed with null marrow into wild type recipient mice with wild type niche), we terminated the experiment. Third, a possible niche contribution was one of the reasons for performing the in vitro analysis (LTC-IC and methylcellulose replating assays) that take null cells out of the niche and assess the action of the stem cells outside the animal (for LTC-IC on wild type stroma). *Cdc42*$^{-/-}$ mice show no defects in these assays, while Mtg16-null stem cells do.

We also propose to add data showing that we can re-express Mtg16 in null bone marrow cells and complement the defects in LTC-IC assays. If the defects were due to Niche defects, this complementation would not work. Finally, we have yet to find a phenotype in Mtg16$^{-/-}$ murine embryonic fibroblasts. In fact, Mtg16 is expressed at much higher levels in LSK and B cells than any other hematopoietic cells or fibroblasts (Hunt et al., 2011, Supplemental figure 3 and data not shown), whereas Mtg8 is the key MTG family member in fibroblasts. We are currently testing the levels of expression of Mtg16 in bone marrow stromal cells and expect to see little Mtg16 expressed. This assay should not take more than a week or so to complete and could be added to the data in this manuscript.

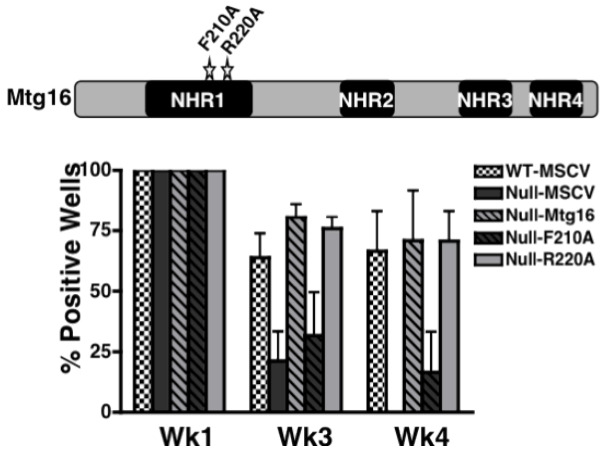

Fig. 5C. Mtg16 re-expression, but not Mtg16(F210A), complements the LTC-IC defect in Mtg16-null bone marrow cells. Schematic diagram shows the positions of the F210A (blocks Mtg8 binding to HEB and Mtg16 suppression of E protein-dependent transcription) and R220A (control mutation that does not affect E protein binding in the crystal structure of Mtg8).

2. A distinction needs to be made between abundance and function of repopulating stem cells. Given the finding that primitive HSCs are reduced >75% in the Mtg16 null bone marrow, a more precise assessment of the function of these HSCs would be obtained through transplantation of equal numbers of sorted wild type and mutant CD45.2 LT-HSCs along with CD45.1 competitor bone marrow. Also, the part of Figure 2B where reconstitution at the zero time point is shown seems unlikely to be an actual experimental measurement, and, if it is not, should be removed from the graph.

While we could certainly perform a competitive bone marrow transplant with purified cells, we would point out that this information is already present in the manuscript, although we did not make it very accessible. That is, by quantifying the defect in the numbers of LSK/CD150+/CD48-/Flt3- cells (Fig. 1), we know that in doing a 10% wild type to 90% null transplant, we have used at least 2 times more null stem cells than wild type (e.g., starting with 25% fewer stem cells yields: 9 X 25% = 225%). The data in Fig. 2 indicates that while we started with over 2 times more null stem cells, we got back only 30-40% for a 5-fold decrease. In addition, in the secondary transplant we started with 30-40% null cells and got back almost nothing for at least a 10-fold decrease (Fig. 3).

We agree that we could have made this quantification more accessible for the readers and will modify the text to make this more explicit including the above calculations and the % of LSK/Flt3- cells that went into the secondary transplant.

We also want to emphasize that the requested experiment is extremely hard on stem cells—coating them with antibodies and forcing them under high pressure as single cells though a laser for sorting. We have already established a defect in Mtg16-null cells in response to proliferative stress, so further stressing these cells may create artifacts. The experiment that we presented accomplishes the same goal, because we know the percentages of LSK/Flt3-/CD150+/CD48- cells in the starting mice for both the primary and secondary transplants, but without the additional stress caused by FACS.

It is also notable that this experiment addresses the niche question to some degree in that the most dramatic phenotype observed is in the secondary transplant, after the null cells have been in the presence of wild type niche for 12 weeks.

3. To identify a defect in maintenance of HSC quiescence it is necessary to study the quiescent fraction of the HSC compartment, the LT-HSCs. For this purpose, the LSK or LSKFlt3- phenotypes are not sufficient. An acceptable phenotype for this purpose would be LSKCD150+CD48- (or even better: LSKCD150+CD48-CD34-). The current data analyze cell populations, which are overwhelmingly composed of short term HSCs with cycling properties completely different from repopulating HSCs. Given that perhaps only 2-3% of LSK cells are true LT-HSCs, a decrease in LSK quiescence from 30 to 25% does not allow any conclusions to be reached regarding the LT-HSC subset.

We would be happy to assess the quiescent status of the LSK/CD150+/CD48- LT-HSC population with the caveat that this population has yet to be proven to be LT-HSC (using limiting dilution transplants) and that the markers suggested is a compilation of 2 separate purification strategies. That is, it is entirely possible that there are 2 or more "stem" cell populations some of which mark with CD150, others that mark with EPCR or other stem cell markers such as Kit and Sca. The vast majority of published papers that look at BrdU incorporation use LSK or LSK/Flt3-.

4. The experiment showing normal homing of Mtg16 null bone marrow cells does not address whether the cells found in the bone have a stem cell phenotype. It should be possible to determine at least the number of LSK cells by flow cytometry. For the colony assays on homed cells, one would of course expect the un-injected background to be zero colonies, but it would probably be appropriate to verify this experimentally.

The reason that FACS is not used to assess homing after transplant is that there is massive apoptosis occurring in the bone marrow due to the lethal irradiation. We attempted this experiment early on in our studies, but it is just impossible to obtain accurate data. One reason is that only 10% of the injected cells home to the marrow (most go to the spleen), so one is assessing only very few cells in the context of millions of cells undergoing apoptosis. After consulting with stem cell experts, we followed their advice to use methylcellulose assays, which yielded very clear results. As the reviewer suggests, the controls yield no colonies. We will add this description to the text.

5. The failure of Mtg16 null bone marrow to rescue lethally irradiated recipients need not necessarily be due to stem cell failure. The authors have themselves previously shown that Mtg16 null bone marrow shows a myeloid-biased lineage allocation. It is therefore entirely possible that lethality is, at least to some extent, due to failure to adequately reconstitute erythrocytes and platelets, but not a general hematopoietic failure. Analysis of the peripheral blood (erythrocyte parameters, platelet numbers and differential counts would suffice) during the recovery phase would allow the relative kinetics of myeloid and platelet/erythrocyte reconstitution to be assessed.

We assume that this comment is directed towards the data in Fig. 2A and we agree that this lethality is likely due to defects in erythopoiesis and only used these data to lead into the competitive bone marrow transplants (Fig. 2B). Certainly, a stem cell defect would exacerbate these defects, so it is useful to include in the manuscript, and we will revise the text to ensure these points are clear.

6. The analysis of the gene expression data needs to be more systematic and unbiased. For example, is the enrichment of S-phase genes systematic and significant? Gene Ontology or Gene Set Enrichment Analysis-based interpretations would be appropriate. The identification of E2f2 as a putative Mtg16 target in interesting, but no functional data is provided to assess its relevance (shRNA-based lentiviral knockdown in Mtg16 null HSCs, for example, would be fairly straightforward, and should even benefit from a selective advantage of the knockdown cells, if the authors' hypothesis is correct).

We would be happy to provide more of our analysis from Gene Set Enrichment software.

We believe that it is critical not to over interpret the ChIP data for E2F2 and the cyclins. These data do say that E2F2 is a direct target for regulation that requires Mtg16, which is important new information for the field. However, these data do not say that the other genes identified to be up regulated are not directly regulated by Mtg16. That is, negative ChIP data cannot be interpreted to say that these are not targets, only that the region assessed was not occupied by Mtg16. While it would be nice to determine if knocking down E2F2 reverted the phenotype, this seems very unlikely given that over 20 cell cycle control genes are up-regulated in the absence of Mtg16. As such, we do not believe that up-regulation of one of these genes is solely responsible for the loss of quiescence in the Mtg16-null cells. This would require us to knockdown 20 genes individually and together, which is not technically feasible. Finally, we would emphasize that what is really needed is not to eliminate the expression of a key inducer of the S phase (which may also cause problems), but a normalization of E2F2 levels. A 50-60% controlled knockdown is extremely difficult to achieve. This was recognized by reviewer #2 who said "Given the possible interaction of MTG16 with multiple transcriptional partners, definitive mechanistic analysis will likely involve a large amount of work." In fact, we have new data that suggests that the reviewer was absolutely correct in the assessment that more than one pathway may be affected.

Instead of trying to knock down 20 different genes, we have been working on reconstituting Mtg16 expression. We have not yet been able to complement the defect in bone marrow transplantation assays, but we have been able to complement the phenotypes in LTC-IC assays out to 4 weeks. By comparing wild type Mtg16 to a point mutant (F210A) that fails to suppress E protein-dependent transcriptional activation versus a control mutant (R220A), we show that regulation of E proteins is very important to these phenotypes. However, the F210A mutant did produce some colonies, suggesting that other pathways (perhaps Notch) also contribute. While these data do not directly address E2F2, they do suggest that the effect is dependent on the Mtg16:E protein functional axis, and Mtg16 was found at the E protein binding site in E2F2. We would propose to add these new data as Fig. 5C.

Minor points:

1. Gene nomenclature is highly inaccurate in many places; it is not generally possible to obtain a correct gene name simply by italicizing the protein name (Cyclin D1 = Ccnd1, PU.1 = Sfpi1, Neurofibromatosis-1 = Nf1, C/EBPa = Cebpa, EpoR = Epor, N-Myc = Mycn etc).

We can easily change the nomenclature in the paper, but we used the gene name that most investigators will recognize.

2. For competitively repopulation assays it would be useful if the lineage allocation (B, T, myeloid) of the CD45.1 and CD45.2 fractions could be provided. Since lymphoid and myeloid cells repopulate and decay with very different kinetics differences in lineage allocation may affect the interpretation of the results.

We would be happy to add these data.

3. For the microarray analysis the methodology used for quality control and data processing should be explained, and an accession number for the primary data provided.

We would be happy to expand the description for this methodology.

4. The gating for lack of Flt3 expression seems to include a very small number of cells, compared to what is normally considered Flt3- in the LSK compartment (35-40% of LSK cells).

Though it may look like a small number of cells, the gates for the Flt3- compartment were set to 33% of the LSK cells in the wild type mice. Those same gates were used for the Mtg16-null samples, which had a lower percentage of LSK/Flt3- cells.

5. Indicating the size of gated populations on the flow cytometry plots would be helpful.

We would be happy to provide the percentage of the cells in each gate.

Referee #2

(Remarks to the Author)
In this manuscript, the authors report an essential function for the transcriptional repressor Myeloid Translocation Gene 16 (MTG16) in the maintenance of hematopoietic stem cells (HSCs). A previous paper from this group had described the generation of MTG16-deficient mice (Chyla et al., MCB 2008), as well as specific hematopoietic defects in these mice (erythroid differentiation, bone marrow B lineage cells, CFU-S8, CFU-S12). The current manuscript provides a much more detailed description of the HSC compartment in these mice. They show that Mtg16 loss results in decreased numbers of phenotypically defined HSCs in the bone marrow, markedly decreased radioprotection capacity and decreased long-term reconstitution potential in competitive repopulation assays. Homing to the bone marrow appeared to be preserved, at least when crudely assessed 16 hours after transplantation. In vitro assays showed loss of replating potential and decreased colony formation in LTC-IC assays, suggesting decreased self-renewal. Cell cycle and BrdU incorporation analysis revealed an increased proportion of MTG16-deficient progenitors in S phase and a decrease proportion in G0, suggesting decreased quiescence. Finally, gene expression analysis revealed several upregulated genes that could contribute to the hematopoietic phenotype after loss of MTG16-mediated repression (including N-myc, Cyclin D1, E2F2 and Id family members). In the case of E2F2, this was correlated with ChIP data showing recruitment of both E47 and MTG16 to the E2F2 locus (suggesting that MTG16 could function as a repressor by associating with E proteins at this locus).

Altogether, this is an interesting paper that reports new information about MTG16. In particular, the characterization of HSC defects in MTG16-deficient mice is done very carefully. The authors provide several suggestions regarding the mechanistic effects of MTG16 loss, largely based on gene expression arrays, with more detailed investigation by ChIP of a small subset of candidate targets. Given the possible interaction of MTG16 with multiple transcriptional partners, definitive mechanistic analysis will likely involve a large amount of work.

Specific comments:

1) Figure 1: it would be very useful for the reader if percentages of events were indicated for the gating boxes in the dot plots (even if absolute cell numbers are represented by bar graphs). This applies to other dot plots across the manuscript.

We would be happy to provide the percentage of the cells in each gate.

2) Figure 6C: ChIP analysis is performed in a cell line rather than in primary bone marrow cells. Although there are obviously technical arguments to support this approach, it would be interesting to know if recruitment of

Mtg16 and E47 to the E2F2 locus could be detected in primary cells. Also, can the authors evaluate if Id proteins were also recruited to this locus? This would also have the potential to repress the effects of E proteins, especially given elevated Id1/2 expression in MTG16-deficient cells.

After obtaining the data with the cell line, we did attempt to use lineage negative bone marrow cells with mixed results. In looking over Mtg16 expression, it is highest in LSK and B cell populations with lower levels in progenitor cells. We believe that this mixture of cells creates more background in this assay, so we have to rely on the cell lines for these assays.

3) Figure 7, S5: a central claim in the manuscript is the decreased quiescence of MTG16-deficient long-term HSCs. Thus, it would be very useful if analysis of quiescence and cell cycle activity were performed on as strictly defined populations of HSCs as possible (rather than on heterogenous LSK cells). Fig. S5C is helpful by gating on Flt3- LSK cells and could be shown as a main figure. Even better would be to analyze HSCs as defined in figure 1 using LSK staining and SLAM markers.

We would be happy to attempt this analysis as it is quite fast.

4) Figure S1: although described as showing enhanced myeloid production, the findings could equally be explained by reduced representation of B lineage cells in the MTG16-deficient bone marrow. In other words the increase in myeloid cells could only be relative. The authors need to reword their description. Analysis of lymphoid and myeloid progenitors would also be helpful to further document this phenomenon.

We would be happy to reword this section—the enhanced myeloid production is at the expense of lymphoid cells, so it could be loss of lymphopoiesis as the reviewer suggests.

5) Table S1: upregulated genes include several genes that have been described as Notch target genes (Hes1, Nrarp, Id1, among others). The authors have previously published work showing an interaction between CSL and MTG16. Can the authors elaborate on that? Can some of the gene expression changes observed in MTG16-deficient progenitors be related to loss of CSL/MTG16-mediated repression of Notch target genes?

This is an excellent point. Interestingly, in our analysis of these genes we realized that many Notch targets are also regulated by E proteins and our new data suggest that E proteins are very important in LTC-IC studies (Fig. 5C). We will try to make this point better in our descriptions and discussion.

Referee #3

(Remarks to the Author)
The manuscript by Fisher et al reports the finding that Mtg16 is required to maintain hematopoietic stem cells in quiescence and to control their long term self-renewal capacity. The authors show that Mtg16^{-/-} HSCs fail to reconstitute hematopoiesis both in serial and in competitive transplantation assays whereas their marrow homing capacities remain unaltered, consistent with a cell-intrinsic defect. By BrdU labelling and Hoechst staining, the authors identify a defect in the quiescence state of the LSK population which is enriched in HSC, associated with increased E2f2 expression.
Comments:
In adult mice, hematopoietic stem cells reside in a quiescence state that preserves their long term activity. A number of genes have been shown to control this quiescence state and the authors provide additional evidence for a role of the Mtg16 co-repressor in this process. The work is carefully conducted. Although primary transplantation assays show that Mtg16^{-/-} cells are less competitive and are prematurely exhausted, secondary transplantation is more revealing of long term stem cell activity and the most convincing data come from the assessment of the population of LSK/Flt3- which is severely decreased in secondary

transplantation (Figure 3B). Nonetheless, a number of issues compromise the manuscript in its current form and should be addressed.

1) Given that the read out in transplantation assays is the percentage of donor-derived mature cells and given that the authors have previously shown an important role for Mtg16 in short term stem cells and multipotent progenitors, the question arises whether the decreased competition that the authors report in the present study might be due to decreased short term stem cell/multipotent progenitors or decreased long term stem cells. Indeed, the early lethality of mice transplanted with Mtg16_{-/-} cells at 30-40 days is a clear indication of a failure of short term reconstitution (Figure 2A).

We agree with the reviewer that the straight BMT experiment (Fig. 2A) is likely measuring megakaryocyte/erythroid progenitor cell functions, which is why we proceeded to test the LT-HSC functions in competitive BMT experiments. For these studies, we started the cBMT with 90:10 ratio to ensure at least a 2:1 ratio for long-term stem cells. These data, coupled with the secondary transplant, LTC-IC and methylcellulose replating assays point to a stem cell defect

2) The authors have circumvented this early lethality problem by transplanting a limiting dose of normal bone marrow cells at a ratio of 1:10. This approach clearly solved the lethality problem but does not directly address the question of long term stem cell self-renewal even if normal cells outcompete Mtg16-/- cells, because of the above mentioned possibility that the read out depends not only on long term stem cells but also on progenitors that are continuously generated from these stem cells. Unless the authors can find conditions in which short term stem cells and progenitors are not compromised while long term stem cells are significantly affected, then the conclusions of the study need to take into consideration the confounding issues of short term versus long term stem cell activity.

We recognize that these are difficult points to digest if one does not carefully assess the overall compilation of data. The primary and secondary transplantation experiments are done in the presence of wild type stem cells, progenitor cells and mature cells. Based on our knowledge of fewer LSK/CD150+/CD48-/Flt3- cells in the null marrow, we used a 90%:10% competitive transplant to initiate the study with 2 fold more null stem cells than wild type, but still provide plenty of wild type cells to complement the defects in short term stem cells and progenitor cells. Moreover, the reduction in competitiveness yields a near 1:1 ratio for the secondary transplant. In both cases there are wild type cells present to complement any progenitor cell defects. Instead, the null cells were essentially undetectable after the secondary transplant (Fig. 3B). These competitive transplants are the gold standard for separating progenitor phenotypes from long-term stem cell defects.

3) Along the same line, in vitro serial replating and long-term culture with exhaustion at 3 weeks illustrated in Figure 5 are also compatible with more short term stem cell activity.

We agree with the reviewer that the methylcellulose replating assay could be assessing short term stem cell activity. However, the LTC-IC cultures are specifically designed to detect long-term (12 week) stem cell self-renewal. If stem cells are present, they should continue to produce short-term stem cells and progenitor cells for 12 weeks. In principle, it is possible that the stem cells are present, but cannot make ST-HSCs or progenitor cells, but we have not observed any defects in differentiation to date.

4) The authors show by various approaches that E2f2 is a target of regulation by Mtg16. However, the functional significance of E2f2 upregulation in this study is unclear. Is the phenotype reported here for Mtg16 deficiency rescued by knocking down E2f2?

See Reviewer 1 #6 above.

5) Finally, quiescence monitored in the LSK population shows rather modest variations between Mtg16<sup>+/+</sub> and Mtg16-/- cells. Have the authors monitored cell cycle in subpopulations that are more enriched in long term stem cells, i.e. SLAM/LSK or LSK/Flt3^{neg}?

We did show the LSK/Flt3- population in the paper, but we can also look at the LSK/SLAM population.

1st Editorial Decision 01 September 2011

Thank you for submitting your manuscript for consideration by the EMBO Journal.

After carefully reading your reply to the reviewers' comments, and discussing it with my colleagues and Chief Editor, we decide to invite you to submit a revised version of the manuscript. Provided that you would modify and amend the manuscript as you said you would, we will send it back to reviewers.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. As the revision involves in vivo experiments, please let me know, maybe closer to the 3 month-deadline, if you think you will need an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

1st Revision - authors' response 02 November 2011

Response to the reviewer's comments:

Referees' comments:

Referee #1

(Remarks to the Author)
The authors here use an Mtg16 knockout mouse strain to address the role of Mtg16 in hematopoietic stem cell function. It is observed that HSCs, and in particular immature HSCs (LSKCD150+CD48-), are under-represented by phenotype in Mtg16 null bone marrow. These cells are almost entirely lost during successive competitive transplantations. This is attributed to increased cycling of HSCs, as measured by BrdU incorporation and Hoechst/Pyronin Y staining of LSK cells. Gene expression profiling of wild type and mutant LSK populations identified up-regulation of several positive regulators of cell cycle progression (Mycn, Ccnd1, E2f2) of which E2f2 was found to bind Mtg16 by ChIP analysis.

While the results presented clearly identify a defect in repopulation of HSCs derived from Mtg16 null mice, it is not clear that the conclusion that this is a defect intrinsic to the HSC compartment is fully supported by the data, not is it clear that the cellular defect identified, namely increased cycling, can be attributed to repopulating HSCs. Finally, the putative role of deregulation of cell cycle genes in the observed phenotype is not substantiated by any experimental data.

Major points:

1. A general issue with assessing the role of intrinsic regulators of adult definitive HSC function using a general (as opposed to conditional) knockout is that the effect on the HSCs may be indirect (e.g. through a role for the gene, here Mtg16, in the developmental or adult HSC niche), or developmental (e.g. due to an intrinsic defect in formation of fetal liver HSCs, leading to an exhaustion phenotype in the adult HSCs). Some, but not all, of these concerns could be addressed by the reconstitution of Mtg16 null recipients with wild type bone marrow, and assessing function of HSCs some months post-transplant through re-transplantation into wild-type recipients, which would provide information about the state of the HSC niche in adult Mtg16 null mice. However, the state of the art for this type of experiment is the inactivation of a conditional allele in transplanted bone marrow, whereby developmental and niche phenotypes can be circumvented.

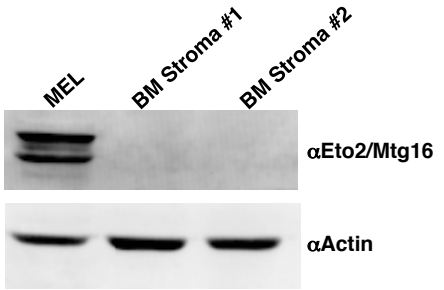

Figure for the reviewers: Immunoblot analysis of Murine Erythroleukemia (MEL) cell or wild type bone marrow stroma cell lysates shows the absence of Mtg16 protein in wild type bone marrow stroma cells. Wild type bone marrow stroma #1 and #2 are biological replicates from two separate preparations of stromal cells isolated from two wild type mice. WT, wild type; BM, bone marrow.

These considerations are not entirely theoretical: while the data are not fully comparable, Mtg16-deficient HSCs do not decrease as a percentage of the total Mtg16 BM population relative to wild type HSCs when transplanted into a wild-type host (Figure 2D), in seeming contrast to what is observed in an Mtg16 null host (Figure 1), indicative of an environmental contribution to the phenotype.

We thank the reviewer for the comment as we were also quite concerned about potential niche contributions and did several experiments to rule this out. We apologize for not making these points more clear. We have added data showing that Mtg16 is expressed only at low levels in stromal fibroblasts as compared to Mtg8 and Mtgr1 using QRT-PCR (about 5- to10 cycles different), making a niche defect unlikely. In fact, Mtg16 is nearly undetectable by western blot (this negative data is not shown, but provided to the reviewers). Moreover, we discuss data that Mtg16-null stem cells do not leave the niche (as compared to the data in the literature for $Cdc42^{-/-}$ mice).

We have also added data showing that we can re-express Mtg16 in null bone marrow cells and complement the defects in LTC-IC assays (new Figure 5C, see below). If the defects were due to Niche defects, this in vitro complementation would not work. In addition, we demonstrate that point mutants that fail to suppress "E protein"-dependent transcription fail to complement the defects observed in LTC-IC assays. These data provide a further link between Mtg16 and E proteins that might regulate cell cycle control genes such as E2F2.

2. A distinction needs to be made between abundance and function of repopulating stem cells. Given the finding that primitive HSCs are reduced >75% in the Mtg16 null bone marrow, a more precise assessment of the function of these HSCs would be obtained through transplantation of equal numbers of sorted wild type and mutant CD45.2 LT-HSCs along with CD45.1 competitor bone marrow. Also, the part of Figure 2B where reconstitution at the zero time point is shown seems unlikely to be an actual experimental measurement, and, if it is not, should be removed from the graph.

We agree that this experimental design can be very useful, especially when comparing different populations of cells from the same mice, but given that Mtg16-null hematopoietic cells do not respond well to different types of stress, we were concerned that they might not be able to handle being coated with antibody and sorted under high pressure by the flow cytometer. Indeed, any defect in the null cells would be hard to interpret (was there a true stem cell defect or a failure to recover from stress?). Rather, we carefully quantified the numbers of early progenitor and stem cells in the null mice prior to competitive transplant to be able to ensure that we were observing a defect in stem cell functions. We apologize for not making this quantification very clear. We have adjusted the text to show the calculations based on the quantification of the defect in the numbers of LSK/CD150+/CD48-/Flt3- cells (25% the normal number, Fig. 1) and using a 10% wild type to 90% null transplant. This yields at least 2 times more null stem cells than wild type in the injected populations of cells (e.g., starting with 25% fewer stem cells yields: 9 X 25% = 225%). The data in Fig. 2 indicates that while we started with over 2 times more null stem cells, we got back only 30-40% for a 5- to 6-fold decrease in stem cell activity. In addition, in the secondary transplant we started with 30-40% null cells and got back almost nothing for at least a 10-fold decrease (Fig. 3).

It is also notable that this experiment addresses the niche question to some degree in that the most dramatic phenotype observed was in the secondary transplant, after the null cells have been in the presence of wild type niche for 12 weeks.

We have modified Fig. 2B as the reviewer suggested.

3. To identify a defect in maintenance of HSC quiescence it is necessary to study the quiescent fraction of the HSC compartment, the LT-HSCs. For this purpose, the LSK or LSKFlt3- phenotypes are not sufficient. An acceptable phenotype for this purpose would be LSKCD150+CD48- (or even better: LSKCD150+CD48-CD34-). The current data analyze cell populations, which are overwhelmingly composed of short term HSCs with cycling properties completely different from repopulating HSCs. Given that perhaps only 2-3% of LSK cells are true LT-HSCs, a decrease in LSK quiescence from 30 to 25% does not allow any conclusions to be reached regarding the LT-HSC subset.

We have added the requested data on the LSK/CD150+/CD48- population, which shows similar changes as compared to the previous LSK/Flt3- data. This is new Figure 7D.

4. The experiment showing normal homing of Mtg16 null bone marrow cells does not address whether the cells found in the bone have a stem cell phenotype. It should be possible to determine at least the number of LSK cells by flow cytometry. For the colony assays on homed cells, one would of course expect the un-injected background to be zero colonies, but it would probably be appropriate to verify this experimentally.

The reason that FACS is not used to assess homing after transplant is that there is massive apoptosis occurring in the bone marrow due to the lethal irradiation. We attempted this experiment early on in our studies, but it is just impossible to obtain accurate data. One reason is that only 10% of the injected cells home to the marrow (most go to the spleen), so one is assessing only very few cells in the context of millions of cells undergoing apoptosis. After consulting with stem cell experts, we followed their advice to use FACS with CSFE and methylcellulose assays, which yielded very clear results.

As the reviewer suggests, the controls yielded very few colonies and following protocols provided by our stem cell colleagues, we subtracted this background from the data before calculating the % Homing. Obviously, we cannot show the background in this context as it is not "% Homing", but we have clarified this issue in the text.

5. The failure of Mtg16 null bone marrow to rescue lethally irradiated recipients need not necessarily be due to stem cell failure. The authors have themselves previously shown that Mtg16 null bone marrow shows a myeloid-biased lineage allocation. It is therefore entirely possible that lethality is, at least to some extent, due to failure to adequately reconstitute erythrocytes and platelets, but not a general hematopoietic failure. Analysis of the peripheral blood (erythrocyte parameters, platelet numbers and differential counts would suffice) during the recovery phase would allow the relative kinetics of myeloid and platelet/erythrocyte reconstitution to be assessed.

We agree that this lethality observed in the non-competitive bone marrow transplant experiment (Fig. 2A) is likely due to defects in erythopoiesis and only used these data to lead into the competitive bone marrow transplants (Fig. 2B). Certainly, a stem cell defect would exacerbate these defects, so it is useful to include the data in Fig. 2A in the manuscript, but this is certainly

only a prelude to the gold standard assay of competitive transplants. We have revised the text to ensure these points are clear.

6. The analysis of the gene expression data needs to be more systematic and unbiased. For example, is the enrichment of S-phase genes systematic and significant? Gene Ontology or Gene Set Enrichment Analysis-based interpretations would be appropriate. The identification of E2f2 as a putative Mtg16 target in interesting, but no functional data is provided to assess its relevance (shRNA-based lentiviral knockdown in Mtg16 null HSCs, for example, would be fairly straightforward, and should even benefit from a selective advantage of the knockdown cells, if the authors' hypothesis is correct).

We extensively analyzed our data, but the analysis presented was a Gene Ontology analysis. The cell cycle group of genes were significantly up-regulated in null cells at $p = 0.006$ for genes changing at least 1.5-fold, and $p = 0.05$ for genes up-regulated by at least two fold. We have clarified the text and thank the reviewer for pointing this out.

When assessing the function of a transcription factor that affects networks of genes, it is exceedingly difficult and rare to assign a phenotype to a single gene. Moreover, we do not want to over interpret the ChIP data for E2F2 and the cyclins. These data show that E2F2 could be a direct target for regulation by Mtg16, but negative ChIP data cannot be interpreted to say that other genes (e.g., Cyclins) are not targets, only that the region assessed was not occupied by Mtg16. While it would be nice to determine if knocking down E2F2 reverted the phenotype, this seems very unlikely given that over 20 cell cycle control genes are up-regulated in the absence of Mtg16. Moreover, we would emphasize that what is really needed is not to eliminate the expression of a key inducer of the S phase (which may also cause problems), but a normalization of E2F2 levels. A 50-60% controlled knockdown is extremely difficult to achieve. This was recognized by reviewer #2 who said "Given the possible interaction of MTG16 with multiple transcriptional partners, definitive mechanistic analysis will likely involve a large amount of work."

Instead of trying to knock down 20 different genes, we have been working on reconstituting Mtg16 expression to address the pathways that are regulated by Mtg16. We have not yet been able to complement the defect in bone marrow transplantation assays, but we have been able to complement the phenotypes in LTC-IC assays (new Fig. 5C). By comparing wild type Mtg16 to a point mutant (F210A) that fails to suppress E protein-dependent transcriptional activation versus a control mutant (R220A), we show that regulation of E proteins is very important to

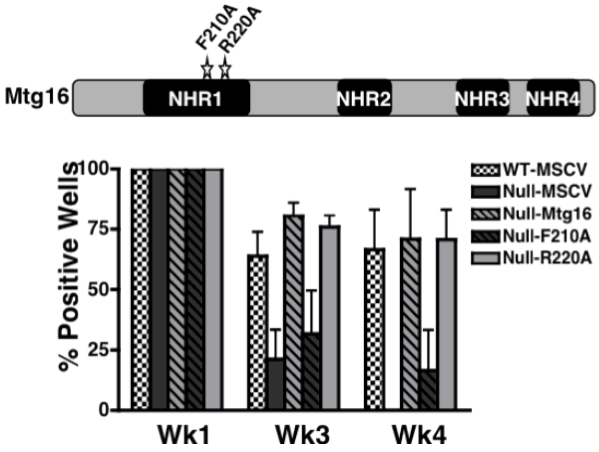

Fig. 5C. *Mtg16* re-expression, but not Mtg16-F210A, complements the LTC-IC defect in *Mtg16*-null bone marrow cells. Schematic diagram shows the positions of the F210A (blocks Mtg8 binding to HEB and Mtg16 suppression of E protein-dependent transcription) and R220A (control mutation that does not affect E protein binding in the crystal structure of Mtg8) point mutants.

these phenotypes. However, the F210A mutant did produce some colonies, suggesting that other pathways (perhaps Notch) also contribute. While these data do not directly address E2F2, they do suggest that the effect is dependent on the Mtg16:E protein functional axis, and Mtg16 was localized by ChIP to the E protein binding site in E2F2. These new data are shown as Fig. 5C.

Minor points:

1. Gene nomenclature is highly inaccurate in many places; it is not generally possible to obtain a correct gene name simply by italicizing the protein name (Cyclin D1 = Ccnd1, PU.1 = Sfpi1, Neurofibromatosis-1 = Nf1, C/EBPa = Cebpa, EpoR = Epor, N-Myc = Mycn etc).

We thank the reviewer for the suggestion and we have added the formal gene name to the description on first use and thereafter we used the gene name that most investigators will recognize as some of the gene names are nonsensical (e.g., p14ARF is called *Cdkn2a* isoform4 even though it has nothing to do with cyclin dependent kinase inhibition).

2. For competitively repopulation assays it would be useful if the lineage allocation (B, T, myeloid) of the CD45.1 and CD45.2 fractions could be provided. Since lymphoid and myeloid cells repopulate and decay with very different kinetics differences in lineage allocation may affect the interpretation of the results.

We agree with the reviewer and have analyzed the bone marrow of the mice after competitive bone marrow transplantation. In addition, we have now provided additional data on lymphoid cells in the supplemental files.

3. For the microarray analysis the methodology used for quality control and data processing should be explained, and an accession number for the primary data provided.

We have expanded the description for this methodology.

4. The gating for lack of Flt3 expression seems to include a very small number of cells, compared to what is normally considered Flt3- in the LSK compartment (35-40% of LSK cells).

Though it may look like a small number of cells, the gates for the Flt3- compartment were set to 30-33% of the LSK cells in the wild type mice. Those same gates were used for the Mtg16-null samples.

5. Indicating the size of gated populations on the flow cytometry plots would be helpful.

We have added the percentage of the cells in each gate.

Referee #2
(Remarks to the Author)
In this manuscript, the authors report an essential function for the transcriptional repressor Myeloid Translocation Gene 16 (MTG16) in the maintenance of hematopoietic stem cells (HSCs). A previous paper from this group had described the generation of MTG16-deficient

mice (Chyla et al., MCB 2008), as well as specific hematopoietic defects in these mice (erythroid differentiation, bone marrow B lineage cells, CFU-S8, CFU-S12). The current manuscript provides a much more detailed description of the HSC compartment in these mice. They show that Mtg16 loss results in decreased numbers of phenotypically defined HSCs in the bone marrow, markedly decreased radioprotection capacity and decreased long-term reconstitution potential in competitive repopulation assays. Homing to the bone marrow appeared to be preserved, at least when crudely assessed 16 hours after transplantation. In vitro assays showed loss of replating potential and decreased colony formation in LTC-IC assays, suggesting decreased self-renewal. Cell cycle and BrdU incorporation analysis revealed an increased proportion of MTG16-deficient progenitors in S phase and a decrease proportion in G0, suggesting decreased quiescence. Finally, gene expression analysis revealed several upregulated genes that could contribute to the hematopoietic phenotype after loss of MTG16-mediated repression (including N-myc, Cyclin D1, E2F2 and Id family members). In the case of E2F2, this was correlated with ChIP data showing recruitment of both E47 and MTG16 to the E2F2 locus (suggesting that MTG16 could function as a repressor by associating with E proteins at this locus).

Altogether, this is an interesting paper that reports new information about MTG16. In particular, the characterization of HSC defects in MTG16-deficient mice is done very carefully. The authors provide several suggestions regarding the mechanistic effects of MTG16 loss, largely based on gene expression arrays, with more detailed investigation by ChIP of a small subset of candidate targets. Given the possible interaction of MTG16 with multiple transcriptional partners, definitive mechanistic analysis will likely involve a large amount of work.

Specific comments:

1) Figure 1: it would be very useful for the reader if percentages of events were indicated for the gating boxes in the dot plots (even if absolute cell numbers are represented by bar graphs). This applies to other dot plots across the manuscript.

We have provided the percentage of the cells in each gate.

2) Figure 6C: ChIP analysis is performed in a cell line rather than in primary bone marrow cells. Although there are obviously technical arguments to support this approach, it would be interesting to know if recruitment of Mtg16 and E47 to the E2F2 locus could be detected in primary cells. Also, can the authors evaluate if Id proteins were also recruited to this locus? This would also have the potential to repress the effects of E proteins, especially given elevated Id1/2 expression in MTG16-deficient cells.

After obtaining the data with the cell line, we did attempt to use lineage negative bone marrow cells with mixed results. In looking over Mtg16 expression, it is highest in LSK and B cell populations with lower levels in progenitor cells (Hunt et al., 2011). We believe that this mixture of cells creates more background in this assay, so we had to rely on the cell lines for these assays.

3) Figure 7, S5: a central claim in the manuscript is the decreased quiescence of MTG16-deficient long-term HSCs. Thus, it would be very useful if analysis of quiescence and cell cycle activity were performed on as strictly defined populations of HSCs as possible (rather than on heterogeneous LSK cells). Fig. S5C is helpful by gating on Flt3- LSK cells and could be shown as a main figure. Even better would be to analyze HSCs as defined in figure 1 using LSK staining and SLAM markers.

We have added the analysis of BrdU incorporation in LSK/SLAM cells as requested.

4) Figure S1: although described as showing enhanced myeloid production, the findings could equally be explained by reduced representation of B lineage cells in the MTG16-deficient bone marrow. In other words the increase in myeloid cells could only be relative. The authors need to reword their description. Analysis of lymphoid and myeloid progenitors would also be helpful to further document this phenomenon.

We have added the requested analysis of lymphopoiesis and reworded this section and thank the reviewer for pointing this out.

5) Table S1: up-regulated genes include several genes that have been described as Notch target genes (Hes1, Nrarp, Id1, among others). The authors have previously published work showing an interaction between CSL and MTG16. Can the authors elaborate on that? Can some of the gene expression changes observed in MTG16-deficient progenitors be related to loss of CSL/MTG16-mediated repression of Notch target genes?

This is an excellent point. Interestingly, in our analysis of these genes we realized that many Notch targets are also regulated by E proteins and our new data suggest that E proteins are very important in mediating the action of Mtg16 in LTC-IC assays (Fig. 5C). We have emphasized this point in our descriptions and discussion.

Referee #3

(Remarks to the Author)
The manuscript by Fisher et al reports the finding that Mtg16 is required to maintain hematopoietic stem cells in quiescence and to control their long term self-renewal capacity. The authors show that Mtg16^{-/-} HSCs fail to reconstitute hematopoiesis both in serial and in competitive transplantation assays whereas their marrow homing capacities remain unaltered, consistent with a cell-intrinsic defect. By BrdU labelling and Hoechst staining, the authors identify a defect in the quiescence state of the LSK population which is enriched in HSC, associated with increased E2f2 expression.
Comments:
In adult mice, hematopoietic stem cells reside in a quiescence state that preserves their long term activity. A number of genes have been shown to control this quiescence state and the authors provide additional evidence for a role of the Mtg16 co-repressor in this process. The work is carefully conducted. Although primary transplantation assays show that Mtg16^{-/-} cells are less competitive and are prematurely exhausted, secondary transplantation is more revealing of long term stem cell activity and the most convincing data come from the

assessment of the population of LSK/Flt3- which is severely decreased in secondary transplantation (Figure 3B). Nonetheless, a number of issues compromise the manuscript in its current form and should be addressed.

1) Given that the read out in transplantation assays is the percentage of donor-derived mature cells and given that the authors have previously shown an important role for Mtg16 in short term stem cells and multipotent progenitors, the question arises whether the decreased competition that the authors report in the present study might be due to decreased short term stem cell/multipotent progenitors or decreased long term stem cells. Indeed, the early lethality of mice transplanted with Mtg16_{-/-} cells at 30-40 days is a clear indication of a failure of short term reconstitution (Figure 2A).

We agree with the reviewer that the straight BMT experiment (Fig. 2A) is likely measuring megakaryocyte/erythroid progenitor cell functions and have revised this text to make this point more clearly. In fact, this is why we proceeded to test the LT-HSC functions in competitive BMT experiments. For these studies, we started the cBMT with 90:10 ratio to ensure at least a 2:1 ratio for null to wild type long-term stem cells. These data, coupled with the secondary transplant, LTC-IC and methylcellulose replating assays point to a stem cell defect.

2) The authors have circumvented this early lethality problem by transplanting a limiting dose of normal bone marrow cells at a ratio of 1:10. This approach clearly solved the lethality problem but does not directly address the question of long term stem cell self-renewal even if normal cells outcompete Mtg16-/- cells, because of the above mentioned possibility that the read out depends not only on long term stem cells but also on progenitors that are continuously generated from these stem cells. Unless the authors can find conditions in which short term stem cells and progenitors are not compromised while long term stem cells are significantly affected, then the conclusions of the study need to take into consideration the confounding issues of short term versus long term stem cell activity.

We recognize that these are difficult points to digest and it is important to look at the entire design of the paper to separate out long-term versus short-term stem cell functions. The primary and secondary competitive transplantation experiments were done in the presence of wild type stem cells, progenitor cells and mature cells. Based on our knowledge of fewer LSK/CD150+/CD48-/Flt3- cells in the null marrow, we used a 90%:10% competitive transplant to initiate the study with 2 fold more null stem cells than wild type, yet still provide plenty of wild type cells to complement the defects in short term stem cells and progenitor cells. Indeed, the 10% wild type cells were sufficient to provide radioprotection after transplant. It is also notable that for the secondary transplant there was closer to a 1:1 ratio of wild type to null cells. Thus, in both cases there are wild type cells present to complement any short-term stem cell or progenitor cell defects. These competitive transplants are the gold standard for separating progenitor phenotypes from long-term stem cell defects. Moreover, rather than simply assess the numbers of downstream cells in the peripheral blood, we analyzed the bone marrow of these mice and found a large (5-10-fold) defect in LSK/Flt3- cells, which is consistent with a stem cell defect.

3) Along the same line, in vitro serial replating and long-term culture with exhaustion at 3 weeks illustrated in Figure 5 are also compatible with more short term stem cell activity.

We agree with the reviewer that in isolation the methylcellulose replating and LTC-IC assays could be assessing short-term stem cell activity. But when coupled with the cBMT results, the data argue for a stem cell defect. We would emphasize that each of the assays used provides a further clue to the defects observed. These in vitro assays take the stem cells out of the niche so they are designed to assess stem cell functions that cannot be derived by in vivo assays. For instance, the LTC-IC cultures are specifically designed to detect long-term (12 week) stem cell self-renewal. If stem cells are present, they should continue to produce short-term stem cells and progenitor cells for 12 weeks. In principle, it is possible that the stem cells are present and self renewing, but cannot make ST-HSCs or progenitor cells, but we have not observed any defects in differentiation to date and this explanation is at odds with the in vivo analysis which show an erosion rather than an accumulation of LSK/SLAM cells. It is also notable that the stem cells appear to produce progenitor cells for 2-3 weeks, albeit at lower numbers. Thus, when considered in the context of the competitive transplants and the in vivo analysis of stem cell numbers, the methylcellulose assays and the LTC-IC assays strongly suggest a stem cell defect that is likely due to a failure to self renew.

4) The authors show by various approaches that E2f2 is a target of regulation by Mtg16. However, the functional significance of E2f2 upregulation in this study is unclear. Is the phenotype reported here for Mtg16 deficiency rescued by knocking down E2f2?

Rather than knocking down a lone gene in the face of a large number of transcriptional changes, we have complemented the LTC-IC defect with wild type or the F210A mutant that cannot regulate E protein targets such as E2F2 and show that Mtg16 must regulate E protein-dependent transcription to fully complement these defects.

5) Finally, quiescence monitored in the LSK population shows rather modest variations between Mtg16<sup>+/+</sub> and Mtg16-/- cells. Have the authors monitored cell cycle in subpopulations that are more enriched in long term stem cells, i.e. SLAM/LSK or LSK/Flt3^{neg}?

We have added the requested LSK/SLAM analysis to our previous LSK/Flt3- analysis.

Pre-Acceptance letter 14 December 2011

Thank you for having submitted your revised manuscript to The EMBO Journal. I have now received the second round of reviews that I am copying below.

As you will see, two of the referees are still raising some issues that I believe could be addressable in writing. The in vivo experiment mentioned by Referee #1 will not be necessary. However, I would really appreciate if you could moderate some of the statements and claims when suggested by referees #1 and #3 in a revised text. In addition, please make sure that the LTC-IC assays are well explained and detailed.

There is no need to provide a point-by-point letter, only the modified manuscript in a .doc format (that you can attach by replied email).

I thank you once more for your interest and support in The EMBO Journal, and I am looking forward to reading the final version of your interesting manuscript.

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1:

The authors have now added additional experiments to support their conclusions. Some of these provide clear improvements. However, I am not convinced that these in all cases actually address the concerns raised. In particular, the results obtained from LTC-IC assays, as currently described, could be misleading, and this aspect would need to be improved before publication.

Point 1:
Regarding the possibility of a niche defect, it is of course encouraging that Mtg16 mRNA levels are low in the bone marrow stroma. However, the actual HSC niche cells are a very small proportion of those analyzed, so these data do not in any way rule out that Mtg16 is important for the niche; this can only be determined by actual functional studies.

The authors also have performed what they describe as LTC-IC assays to evaluate the ability of virally transduced Mtg16 to rescue HSC function. These experiments are, however, very poorly described (how many cells were plated/well? How were positive wells scored? What are the numbers of cobblestone areas/well? etc.), and do not in their present form support the proposed conclusion. Again, the preferred functional readout here is in vivo reconstitution, which should be well within the capability of the authors.

Point 2
The new transplantation experiments more clearly show the HSC defect, and are an improvement. It is, however, not correct to state that the more severe phenotype in secondary recipients is indicative of an intrinsic defect. The same can be observed with HSCs from e.g. Wnt3-/- mice, where the defect is clearly extrinsic.

Point 3
Absent the actual FACS profiles the quality of these data cannot be properly evaluated, but the numbers provided are consistent with a LT-HSC defect.

Point 4
Others have been able to quantify HSCs after homing, so this argument is not entirely valid. In addition, the CFU assays are not an appropriate proxy for stem cell activity. Finally, it is critical that

the CFU background values are shown, as their size relative to those seen upon transplantation is important to evaluate the reliability of the data obtained.

Point 5
OK with clarification.

Point 6
The clarification regarding the bioinformatics is OK. As discussed above, the LTC-IC assays are too poorly described to allow their relevance to be assessed.

Referee #2:

The authors have adequately addressed my concerns in their revised manuscript.

Referee #3:

Fischer et al have re-submitted a revised version of the manuscript entitled "Myeloid Translocation Gene 16 is required for maintenance of hematopoietic stem cell quiescence". The revised version includes a rescue of Mtg16-/- cells by wild type but not Mtg16-F210A mutant, which significantly improves the manuscript, as well an assessment of HSCs based on two additional markers, CD150+CD48- and BrdU labelling for cell cycle. The manuscript conveys new information on the importance of Mtg16 in maintaining HSC quiescence, and this message is strengthened by new data, although there are still weaknesses.

The authors show that in the absence of Mtg16, there are less LSK FLT3-CD150+CD48- and bone marrow cells from Mtg16-/- mice are deficient in reconstitution assays in the short term (7 weeks) as well as in the long term (52 weeks). These cells are also less competitive than wild type cells to reconstitute the LSK Flt3- (HSC-enriched) population in transplanted mice. Finally, Mtg16-deficient cells are also at a disadvantage in CFU-C replating assays and in LTC-IC assays. These data are unquestionable and show a stem cell defect as well as defects in non-stem cell populations. The difficulty here in dissociating the functional importance of Mtg16 in HSC and in non-stem cell populations can be seen at several levels. The radiation protection assay shown in Figure 2A in which transplanted mice are unable to survive beyond 30 days (low cell dose) or 50 days (high cell dose) clearly reveals a failure of short term protection, which is not due a stem cell defect and should be acknowledged as such. A better indication of stem cell activity comes from data shown in Figure 2D in which these cells are less competitive in reconstituting the LSK Flt3- pool in transplanted mice. There is also an important discrepancy between the rather modest effect of Mtg16-deficiency on stem cell quiescence, as assessed by BrdU incorporation in the LSK CD150+CD48- population (8% in wild type versus 11% in Mtg16-/- cells, Figure 7D, a significant albeit modest difference) and the dramatic failure in short term radio-protection assay (Figure2A) as well as the dramatic decrease in CFU-C replating or in LTC-IC (Figures 5A, 5B). In their rebuttal letter, the authors bring forth several arguments which are mostly valid. Nonetheless, given all the technical limitations highlighted by referee1 as well as my own previous comments, the abstract and the text should make less definitive statements about the requirement for Mtg16 in stem cell self-renewal and quiescence. Finally, a 2h BrdU injection is a very short pulse to label quiescent stem cells. Typically, BrdU administration is done over at least 2 days if not longer in stem cell studies. Additionally, the authors should monitor Hoechst and Pyronin staining in the LSK CD150+CD48- since stem cell quiescence is a major aspect of the study.

As a minor point, LSK is a common abbreviation for Lin-Sca+Kit+. However, SLAM is a more generic abbreviation for a family of 'signalling lymphocyte activation molecules' which is not restricted to CD150 and CD48. Therefore, CD150 and CD48 should be used instead of SLAM.

